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Effect of Obesity on the Skeletal Muscle Angiogenic Response Following Acute Resistance Exercise

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Entitled

EFFECT OF OBESITY ON THE SKELETAL MUSCLE ANGIOGENIC RESPONSE FOLLOWING ACUTE RESISTANCE EXERCISE

For the degree of Master of Science

Is approved by the final examining committee:

Timothy Gavin
Chair

Bruno Tesini Roseguini

Tara M. Henagan

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Approved by Major Professor(s): Timothy Gavin

Approved by: David B. Klenosky 6/30/2016

Head of the Departmental Graduate Program Date

EFFECT OF OBESITY ON THE SKELETAL MUSCLE ANGIOGENIC RESPONSE FOLLOWING ACUTE RESISTANCE EXERCISE

A Thesis

Submitted to the Faculty

of

Purdue University

by

Jessica A Weiss

In Partial Fulfillment of the

Requirements for the Degree

of

Master of Science

August 2016

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LIST OF ABBREVIATIONS

NHLBI	National Heart, Lung, and Blood Institute
T2DM	Type II diabetes mellitus
NIDDK	National Institute of Diabetes and Digestive and Kidney Diseases
EC	Endothelial cells
VEGF	Vascular endothelial growth factor
eNOS	Endothelial nitric oxide synthase
NO	Nitric oxide
TSP-1	Thrombospondin-1
AMPK	AMP-activated protein kinase
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
AMP	Adenosine monophosphate
p-AMPK	phosphorylated AMPK
MAPK	Mitogen-activated protein kinase
BMI	Body mass index
LN	Lean
OB	Obese
PAR-Q	Physical activity readiness questionnaire

VO_{2MAX}	Maximal oxygen consumption
1RM	1 repetition maximum
RPE	Rate of perceived exertion
RER	Respiratory exchange ratio
HDL	High density lipoprotein
LDL	Low density lipoprotein
FSG	Fasting Serum Glucose
FSI	Fasting Serum Insulin
ANOVA	Analysis of variance

ABSTRACT

Weiss, Jessica A. M.S., Purdue University, August 2016. Effect of Obesity on the Skeletal Muscle Angiogenic Response Following Acute Resistance Exercise. Major Professor: Timothy Gavin.

With the rates of obesity remaining high, health risks are abundant. This includes an increased possibility of the development of insulin resistance and type 2 diabetes mellitus (T2DM). Several biochemical and histochemical characteristics associated with obesity have been reported, including a reduction in capillary density in the skeletal muscle and increased muscle fiber size. Resistance exercise is known to induce hypertrophy and increase skeletal muscle capillarization. Following acute resistance exercise, it is hypothesized angiogenic factors are reduced and angiostatic factors are increased in the skeletal muscle of obese individuals. Muscle biopsies from the vastus lateralis were obtained to investigate whether VEGF, VEGF receptor, TSP-1 expression, and AMPK phosphorylation (an indicator AMPK activation) are lower in obese compared to lean individuals at rest and in response to an acute bout of exercise. Muscle biopsies were taken before, 15 MIN, and 3 HR following 3 sets of single-leg knee extension at 80% 1 RM. Eight sedentary lean (4 male, 4 female) and eight sedentary obese (4 male, 4 female) individuals participated in this study. No differences in VEGF or VEGF receptor mRNA and protein levels were found at rest or following exercise in lean or obese individuals. Exercise increased VEGF mRNA independent of group. Resistance exercise did not affect TSP-1 mRNA and protein expression or AMPK

phosphorylation and no differences between lean and obese were found. The present study suggests that the angiogenic response to resistance exercise (a known hypertrophic stimulus) is similar in lean and obese individuals.

CHAPTER 1. INTRODUCTION

1.1 Obesity and Insulin Resistance

The prevalence of obesity in the United States remains high, with 78.6 million adults currently falling under this classification (Centers for Disease Control and Prevention, 2014). Many chronic conditions are associated with obesity, which has earned it the title of the second leading cause of preventable death in the United States by the National Heart, Lung, and Blood Institute (NHLBI). Health problems associated with obesity include hypertension, heart disease, metabolic syndrome, and type 2 diabetes mellitus (T2DM) (Mayo Clinic, 2015). In 2008, the estimated annual medical cost of obesity in the United States was $147 billion and has been projected to rise due to the increasing prevalence of T2DM (Centers for Disease Control and Prevention, 2014). According to the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), T2DM frequently is the result of prolonged insulin resistance or a decreased sensitivity and/or responsiveness to insulin. Insulin resistance is common among the obese population. It is estimated that 24% of obese adults in the United States are insulin resistant while in the obese adolescent population prevalence is estimated at 52% (Lee et al, 2006; McKeown et al, 2004).

The human body consists of roughly 40% skeletal muscle, which is responsible for 75% of insulin-induced glucose disposal (Reaven & Laws, 1999).

Several biochemical and histochemical characteristics within the skeletal muscle have been associated with obesity. Studies have shown fiber type dissimilarities between the skeletal muscles of lean compared to obese individuals. A decrease in Type I, slow twitch oxidative, fibers concurrent with an increase in Type IIB, fast twitch glycolytic, fibers has been noted in obese individuals (Lillioja et al, 1987). The area of skeletal muscle fibers of obese individuals is significantly larger than the skeletal muscle fibers of lean individuals (Gavin et al, 2005). Reduced mitochondrial function, reducing the oxidative capacity of the skeletal muscle, has also been reported in obese individuals (Torgan et al, 1989). Primarily, obesity has been strongly correlated to a reduction in capillary density, or the number of capillaries per unit cross-sectional area of muscle (Gavin et al, 2005; Lash et al, 1989; Lillioja et al, 1987; Shono et al, 1999; Torgan et al, 1989). Capillaries are essential for the delivery of oxygen and other nutrients important for energy production. Increased capillarization ensures adequate diffusion capacity across the skeletal muscle (Hudlicka, Brown & Egginton, 1992). Therefore, alterations in skeletal muscle capillarization may contribute to much of the insulin resistance associated with obesity (Lillioja et al, 1987; Shono et al, 1999).

1.2 The Impact of Exercise

Exercise has been proven to be beneficial to overall health in a variety of ways. Those who participate in physical activity for 7 hours or more a week reduce their risk of mortality by 40% (Centers for Disease Control and Prevention, 2015). It has been well-established that endurance training provides many improvements to overall health. A host of effects of aerobic exercise support improvements which include cardiac and skeletal

muscle changes. Adaptations in skeletal muscle include increases in mitochondrial content, respiratory capacity, and capillary density of muscle fibers (Andersen & Henriksson, 1977; Hermansen & Wachtlova, 1971; Holloszy & Coyle, 1984; Yamashita et al, 1993). Regular resistance exercise can also improve cardiovascular, bone, and muscle health and reduce the risks of many chronic diseases (American College of Sports Medicine Position Stand, 1990). Muscle hypertrophy observed with resistance exercise contributes to an increase in muscle strength, basal metabolic rate, and lean body mass, while decreasing body fat composition. Resistance exercise has also been shown to benefit glucose metabolism, lowering basal insulin levels and increasing insulin sensitivity (Pollock & Vincent, 1996). Less recognized as an outcome of resistance exercise is an increase in skeletal muscle capillarization (McCall et al, 1996; Schantz, 1982). Increased capillary density improves the exchange of oxygen, substrates, and metabolites between blood and skeletal muscle (Andersen & Henriksson, 1977; Hermansen & Wachtlova, 1971; Poole, Copp, Ferguson, & Musch, 2013; Tesch, Thorsson & Kaiser, 1984), which is vitally important for individuals with chronic conditions where this exchange has been compromised (Adair & Montani, 2010).

1.3 Skeletal Muscle Angiogenesis

Angiogenesis, the process of generating new capillary blood vessels, occurs in response to signals from changes in mechanical stimuli and metabolic demand (Hudlicka, Brown & Egginton, 1992). It involves a complex cascade of events leading to the migration and proliferation of capillary endothelial cells (EC) (Engerman et al, 1967).

The interplay of both angiogenic (positive) and angiostatic (negative) factors contribute to this process.

Angiogenic and Angiostatic Factors

One of the most vital pro-angiogenic factors for basal and exercise-induced capillarization is vascular endothelial growth factor (VEGF). VEGF is a 45 kDa heparin-binding vascular EC mitogen of the micro- and macrovascular EC of arteries, veins, and the lymphatics (Amaral et al, 2001; Dvorak et al, 1979; Ferrara & Davis-Smyth, 1997; Ferrara & Henzel, 1989; Gavin TP, 2009; Olfert et al, 2009; Tang et al, 2004; Wagner et al, 2006). Although several different isoforms exists, VEGF-A plays the most crucial role in exercise-induced skeletal muscle angiogenesis (Shweiki et al, 1992; Waltenberger et al, 1994). It is released into the muscle interstitial space in response to mechanical stimuli and other stress inducing factors (Gavin et al, 2007; Hoffner, Nielsen, Langberg, & Hellsten, 2003; Hoier et al, 2011; Hoier et al, 2012; Hoier et al, 2010; Ouchi, Shibata, & Walsh, 2005), including hypoxia, oxidative stress, growth factors, and cytokines (Ikeda et al, 1995; Kuroki et al, 1996; Takahashi et al, 2002; Wang, Huang, Kazlauskas, & Cavenee, 1999).

Two receptors identified as having a high affinity for VEGF are VEGFR1 (de Vries et al, 1992) and VEGFR2. Expression of both receptors is restricted to the vascular endothelium, where the positive charge of the surface of VEGF facilitates its binding (Ferrara & Keyt, 1997; Keyt et al, 1996; Yamaguchi et al, 1993). VEGFR2 is suggested to be the receptor principally involved in angiogenesis (Millauer et al, 1993; Gavin TP, 2009; Waltenberger et al, 1994). The phosphorylation of VEGFR2 initiates several signaling cascades, resulting in increased vascular permeability and the migration,

proliferation, and survival of EC (Connolly et al, 1989; Koch et al, 2011). One important cascade involves VEGFR2 activation of endothelial nitric oxide synthase (eNOS) to produce nitric oxide (NO) which in turn stimulates EC growth and motility (Ziche et al, 1994). The NO cascade activated by VEGFR2 phosphorylation is inhibited by thrombospondin-1 (TSP-1) (Isenberg et al, 2006).

TSP-1 is a 450 kDa multifunctional homotrimeric matrix glycoprotein that is thought to play an important role in the regulation of angiogenesis (Iruela-Arispe et al, 1995; Lawler, Slayter, & Coligan, 1978). Though TSP-1 has been shown to stimulate the growth and migration of smooth muscle cells (BenEzra, Griffin, Maftzir, & Aharonov, 1993; Lawler, 2000; Nicosia & Tuszynski, 1994). It prevents angiogenesis via inhibition of VEGF release, VEGF signal transduction, and by direct interaction with VEGF (Iruela-Arispe, Bornstein, & Sage, 1991; Lawler & Lawler, 2012). VEGF bioavailability is decreased due to the influence of TSP-1 on the structure of the extracellular matrix (Iruela-Arispe et al, 1995; Lawler, 1986; Chen, Herndon, & Lawler, 2000). TSP-1 binding to receptors CD36 and CD47 inhibits VEGFR2 phosphorylation (Primo et al, 2005), which hinders EC association at the site of development (Bagavandoss & ilks, 1990; Dawson et al, 1997), migration of EC, and stimulates apoptosis (Iruela-Arispe, Bornstein, & Sage, 1991). Further, the uptake and clearance of VEGF from the extracellular space is induced by TSP-1 binding directly to VEGF (Gupta et al, 1999; Greenaway et al, 2007; Lawler & Lawler, 2012). Consistent with TSP-1 being an angiostatic factor, skeletal muscle capillarization is lower in TSP-1 knock-out (KO) compared to wildtype (WT) mice (Malek & Olfert, 2009).

Intracellular Regulation

A key player in the regulation of the responses to angiogenesis-inducing stressors is the heterotrimeric enzyme 5'-AMP-activated protein kinase (AMPK) (Hardie, 2003). When two molecules of adenosine diphosphate (ADP) are used to produce energy in the form of adenosine triphosphate (ATP), the result is the production of one molecule of adenosine monophosphate (AMP) (Kalckar, 1943). In order for a cell to maintain a necessary level of energy for proper function, the ratio of ATP to AMP must remain high. When reductions in the ratio occur, AMPK is activated. Reduced ATP:AMP is caused by disruptions in the production of ATP, hypoxia or ischemia, and/or increases in the consumption of ATP through muscle contraction (Kudo et al, 1995; Marsin et al, 2000; Winder & Hardie, 1996). The phosphorylation of upstream kinases induces AMPK to regulate cellular energy usage (Hawley et al, 1995). AMP regulates the activation of AMPK and inhibits AMPK dephosphorylation by protein phosphatases (Hawley et al, 1995).

The activation of AMPK stimulates energy-conserving/producing pathways to increase while simultaneously decreasing energy-consuming pathways. Previous research suggests AMPK regulates VEGF in the skeletal muscle through the activation of p38 mitogen-activated protein kinase (MAPK) (Pages et al, 2000; Ouchi, Shibata, & Walsh, 2005; Winzen et al, 1999). AMPK also plays a role in the mediation of NO activity (Zhu & Smart, 2005). Increased AMPK activity has been shown to increase VEGF mRNA and VEGF protein, thereby increasing capillarization and the inactivation of AMPK reduces capillarization at the basal level (Ouchi et al, 2005; Zwetsloot, Westerkamp, Holmes & Gavin, 2008). However, AMPK activation is not necessary for aerobic

exercise-induced angiogenesis, but may play a role in response to resistance exercise (Zwetsloot, Westerkamp, Holmes & Gavin, 2008).

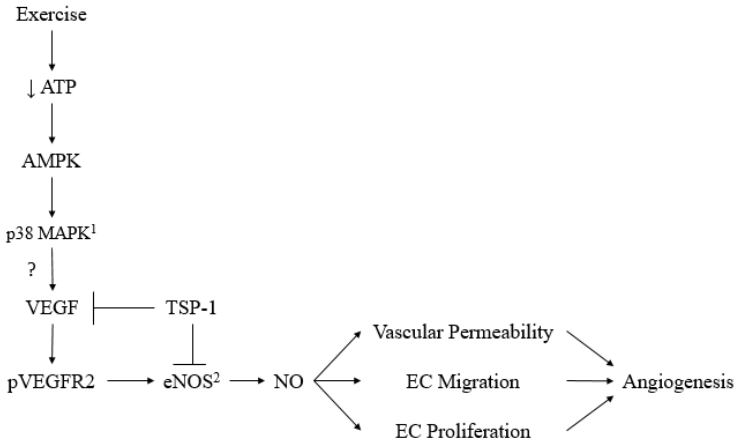

Figure 1: Angiogenic Pathway in Response to Exercise.[1]Ouchi, Shibata & Walsh, 2005-*will not be investigated in this report* [2]Ziche et al, 1994; Isenberg et al, 2006-*will not be investigated in this report*

1.4 Obesity

The skeletal muscle fiber size of obese individuals is markedly increased compared to their lean counterparts (Gavin et al, 2005). VEGF is a necessary component for muscle hypertrophy (Huey, Smith, Sulaeman & Breen, 2016), and basal regulation of skeletal muscle VEGF and capillarization is altered by resting levels of AMPK (Zwetsloot, Westerkamp, Holmes, & Gavin, 2008). However, resting levels of metabolic and regulatory factors of angiogenesis are similar between lean and obese individuals of similar physical inactivity. No differences at rest in AMPK, VEGF, or VEGFR2 expression have been found despite reduced capillary density and muscle fiber hypertrophy observed in obese subjects (Gavin et al, 2005; Steinberg et al, 2004). Though comparisons of basal TSP-1 in the skeletal muscle of lean and obese have not been examined, elevated basal TSP-1 protein and gene expression in adipose tissue resulting in

increased circulating TSP-1 in obese subjects has been noted (Abu-Farha, 2013; Hida et al, 2000; Inoue et al, 2013; Varma et al, 2008; Voros et al, 2005).

1.5 Exercise and Angiogenesis

Aerobic Exercise

Angiogenesis in response to aerobic exercise in lean subjects has been well researched. The interplay of angiogenic and angiostatic factors lead to an increase in capillary density of the skeletal muscle following endurance training (Andersen & Henriksson, 1977). Studies consistently report elevations in VEGF and VEGFR2 expression in the skeletal muscle in response to acute endurance exercise (Benoit et al, 1999; Gavin et al, 2005; Gustafsson et al, 2005; Ryan et al, 2006). Furthermore, TSP-1 is temporarily increased following acute aerobic exercise, appearing to modulate capillary formation (Hoier et al, 2012; Olfert et al, 2006; Olfert, Wagner, & Power, 2000). Cellular energy usage is regulated through an increase of AMPK activity, heavily dependent upon the duration and intensity of the exercise session (Fujii et al, 2000; Rasmussen & Winder, 1997; Stephens et al, 2002; Winder & Hardie, 1996; Wojtaszewski et al, 2000). AMPK is not a necessary part of the angiogenic response to aerobic exercise (Zwetsloot, Westerkamp, Holmes, & Gavin, 2008) but is one potential regulator of VEGF expression, a key component for the muscle hypertrophic response to resistance exercise (Huey, Smith, Sulaeman & Breen, 2016).

Resistance Exercise

There is very little research regarding the skeletal muscle angiogenic response following resistance exercise. However, it is known that resistance training increases

capillarization in the skeletal muscle in lean subjects (McCall et al, 1996; Schantz, 1982). The increased capillarization is in proportion to the hypertrophy of the muscle fibers in order to maintain the capillary density of the skeletal muscle (McCall et al, 1996; Schantz, 1982). The angiogenic response to resistance exercise in terms of VEGF and VEGFR2 expression is similar to that seen following aerobic exercise (Doyle & Haas, 2010; Huey, Smith, Sulaeman & Breen, 2016; Degens, Moore, & Alway, 2003; Gavin et al, 2007; Parvaresh et al, 2010). Increases in VEGF mRNA are observed 2-3 h post resistance exercise (Gavin et al, 2007; Silvennoinen et al, 2015) and VEGFR2 mRNA 4 h post resistance exercise (Gavin et al, 2007). These increases in VEGF and VEGFR2 expression has been shown to play important roles in the maintenance of capillarization, which is necessary for proper hypertrophy of the muscle (Huey, Smith, Sulaeman & Breen, 2016). Elevated AMPK activity has also been noted in lean individuals during and 1 h following resistance exercise (Dreyer et al, 2006), indicating a potential role in regulating VEGF production following resistance exercise. No investigations into TSP-1 expression in the skeletal muscle following resistance exercise have been published.

1.6 Obesity, Exercise, & Angiogenesis

Although no differences reside in basal levels of angiogenic factors between lean and obese, obese demonstrate lower capillarization in the skeletal muscle (Gavin et al, 2005). An important stimulator of the angiogenic process is exercise (McCall et al, 1996; Schantz, 1982). Few have reported any differences in the angiogenic response to aerobic exercise between lean and obese individuals (Gavin et al, 2005; Walton et al, 2015). AMPK activity in obese does not change while lean counterparts show an increase in

AMPK activity following endurance exercise, suggesting an impaired response of AMPK to aerobic exercise (Sriwijitkamol et al, 2007). However, VEGF and VEGFR2 expression in response to aerobic exercise is similar between lean and obese individuals (Gavin et al, 2005). Levels of TSP-1, a potent angiostatic factor, in the adipose tissue of obese individuals return to normal following acute exercise (Abu-Farha et al, 2013).

Although the hypertrophic response to resistance training is evident in obese individuals (Donnelly et al, 1993; Geisler et al, 2011), researchers have not compared the skeletal muscle angiogenic response following resistance exercise in lean and obese individuals to date. The lack of differences in VEGF and VEGFR2 expression following aerobic exercise, combined with the increased fiber diameter with subsequent decreased capillarization (Gavin et al, 2005; McCall et al, 1996), suggests a decreased angiogenic response to muscle hypertrophy in the skeletal muscle of obese individuals.

1.7 Hypothesis

At rest, obese subjects will have increased TSP-1 expression compared to lean counterparts. Following acute resistance exercise, obese subjects will have: (i) lower VEGF, VEGF receptor, and AMPK phosphorylation; and (ii) greater TSP-1 expression compared to lean counterparts.

Delimitations

1. Participants were non-smoking and sedentary between the ages 18 and 35. Sedentary specifies participating in physical activity 3 or fewer times per week for 20 minutes or less. One group of individuals were lean with a body mass index (BMI) ≤ 25 kg/m^2 while the other group was classified as obese with a body mass index (BMI) ≥ 30 kg/m^2.

2. Workloads were tailored to each participant by first establishing their 1 repetition maximum (1RM).

CHAPTER 2. METHODS

The Purdue University Institutional Review Board approved the methods and protocol of this study preceding its execution.

2.1 Participant Selection

Eight healthy, sedentary, lean (LN) individuals (4 women; 4 men) and eight healthy, sedentary, obese (OB) individuals (4 women; 4 men) between the ages of 18-35 were recruited to participate in this study. Subjects were nonsmoking and had no known chronic disease in order to be considered healthy enough to participate. Sedentary qualifies as participating in physical activity 3 times or less for 20 minutes or fewer per week. Lean individuals had a body mass index (BMI) < 25 kg/m^2, while obese individuals had a BMI ≥ 30 kg/m^2. Qualified individuals were given both verbal and written descriptions of the study. Subjects provided signed consent prior to the beginning of the study, as well as completed an Activity and Tobacco Questionnaire and a Physical Activity Readiness Questionnaire (PAR-Q). Subjects were given instructions on completing a three day food intake diary for the days prior to the Day 2 procedures. Subject characteristics are listed in Table 1.

Table 1: Subject Characteristics. Values are listed as Mean ± SE. *n* = 8 subjects/group.

	Lean	Obese	*P* Value
Age, yr	21.5 ± 0.5	24.8 ± 1.6	0.09
Height, cm	169.6 ± 2.9	172.8 ± 3.2	0.48
Mass, kg	62.4 ± 2.0	109.9 ± 7.8	<0.01
BMI	21.7 ± 0.8	36.6 ± 1.8	<0.01
VO_{2Max}, ml O_2/kg/min	37.3 ± 2.7	26.1 ± 2.7	0.01
1 RM, kg	27.8 ± 0.7	40.3 ± 4.7	0.03
1 RM, kg/kg	0.45 ± 0.01	0.37 ± 0.04	0.17
Fasting glucose, mM	4.8 ± 0.1	4.9 ± 0.2	0.54
Fasting insulin, µU/ml	11.8 ± 1.3	28.3 ± 4.8	0.01
Fasting HOMA-IR	2.46 ± 0.29	6.38 ± 1.23	0.01
Fasting HDL, mg/dl	58.4 ± 7.3	42.9 ± 5.4	0.08
Fasting LDL, mg/dl	88.6 ± 12.8	108.6 ± 5.2	0.11
Fasting Total Cholesterol, mg/dl	162.8 ± 12.3	180.6 ± 7.0	0.17
Fasting Triglycerides, mg/dl	79.4 ± 7.7	131.6 ± 22.5	0.05

2.2 Experimental Design

Day 1: Subjects reported to the Max E. Wastl Human Performance Laboratory where their height, weight, and dominant leg was recorded prior to the completion of a VO_{2MAX} test. Subjects were then given a 15 minute rest period before the completion of a one repetition maximum (1 RM) test for single leg, knee extension.

Maximal Oxygen Consumption (VO_{2MAX})

Subject VO_{2MAX} was determined two hours post-prandial using a previously described incremental protocol (Kraus et al, 2004) on a cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). A warm up was performed at 50 W for 5 minutes on the cycle ergometer. Immediately following, additional workloads were added every 2 minutes by 25 W until volitional fatigue. Expired air was monitored and recorded via open-circuit spirometry (Truemax 2400, Parvo Metrics, Salt Lake City, UT). Additionally, heart rate (model H1, Polar Electro, Lake Success, NY) and rate of perceived exertion (RPE) was continuously monitored and recorded. Subjects were verbally encouraged to continue as long as possible. Criteria for the establishment of VO_{2MAX} included 1) a heart rate of at least 90% of age predicted maximum (220-age); 2) a respiratory exchange ratio (RER) at or above 1.10; and 3) an identifiable plateau (≤150 mL increase) in VO_2 despite continued increase in workload. At least two of three criteria were met for all subjects.

One Repetition Maximum (1RM)

Maximal knee extension was randomized between subjects using either the dominant or non-dominant leg. Subjects began in a resting position of 90° and were instructed to fully extend their leg undergoing the exercise using a weight of 13.6 kg. Once parallel to the floor, they returned to the initial position and were given a rest for 1 minute. The exercise was repeated with weight increasing by 4.5 kg until the exercised leg was no longer able to reach full extension. Once this point was attained, the previous weight was determined to be the 1RM.

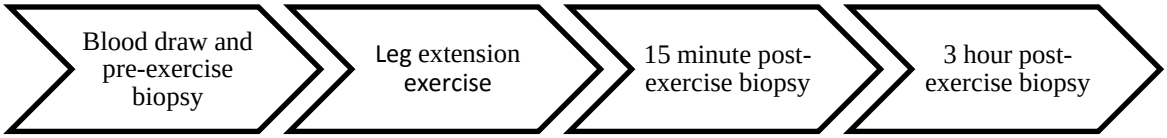

Figure 2: Day 2 timeline

Day 2: Day 2 occurred at least two weeks after Day 1. This was to avoid any influence from the maximal testing incurred on Day 1 on the subjects' performances on Day 2. Three days preceding Day 2, subjects recorded their food intake and partook in a 12 h fast the night prior.

Blood draw

On the morning of Day 2, subjects reported to the Max E. Wastl Human Performance Laboratory where a fasting blood draw was obtained. The 5-8 mL blood sample was used for the measurement of glucose, insulin, high density lipoprotein (HDL), low density lipoprotein (LDL), triglycerides, and total cholesterol. It was collected in a serum tube, allowed for clot formation for 30 minutes at room temperature, and then refrigerated until analysis by Mid-America Laboratories. Insulin resistance was measured as HOMA-IR via the following equation:

$$\text{HOMA-IR} = \frac{FSG * FSI}{22.5 * 18}$$

Fasting serum glucose (FSG) was used in milligrams per deciliter and fasting serum insulin (FSI) was used in microunits per millimeter (Matthews et al, 1985).

Pre- and post-exercise muscle biopsies

Immediately following the blood draw, the subjects underwent the procedure to obtain the first (PRE) of three muscle biopsies. All biopsies were obtained from the vastus lateralis: one biopsy before exercise from the non-working leg and two biopsies from the working leg at 15 min post (15 MIN) and 3 h post (3 HR) exercise. The working leg was randomized between non-dominant and dominant leg between subjects. On the working leg, the two biopsy sites were separated by at least 3 cm (Costill, Pearson, & Fink, 1988). Lidocaine (1%) was used as a local anesthetic to obtain the biopsy samples. Tissue samples were immediately placed in liquid nitrogen and stored at -80°C for future use.

Exercise protocol

Subjects performed three sets of knee extension exercise using their working leg immediately following the first biopsy. Workload was set at 80% of their 1RM determined on Day 1. 8-12 repetitions were performed for the first two sets and subjects were instructed to perform to volitional fatigue for the last set (8-17 repetitions). A two minute rest period was allotted between sets. Preliminary analysis found this protocol to sufficiently fatigue the subjects and completion did not require any outside assistance.

Protein Isolation and Western Blot Analysis

Muscle biopsy samples were frozen in liquid nitrogen and homogenized in RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 0.1% Triton X-100, and 0.5% Deoxycholate) with protease inhibitor cocktail and phosphatase inhibitor (50 mM NaF and 0.2 mM Na_3VO_4). Samples were centrifuged at 4°C for 30 minutes to separate supernatant and insoluble material. The supernatant was used to determine protein concentration by BCA kit (Pierce). Western blot analysis was

performed in order to investigate angiogenic growth factor expression. Angiogenic growth factors include VEGF and VEGF receptor (Gavin et al, 2005); and the anti-angiogenic (angiostatic) factor include TSP-1 (Olfert et al, 2006). AMPK phosphorylation was measured with exercise and obesity as AMPK is an important regulator of VEGF (Zwetsloot, Westerkamp, Holmes, & Gavin, 2008).

Equal amounts of homogenized samples were added to 10% gels. Each membrane was loaded with the 3 HR post exercise sample from subject 4 so that membranes could be compared to one another. Membranes were then run through electrophoresis in running buffer (0.1% SDS, 25 mM Tris, and 190 mM Glycine) at 80 V for 10 minutes, or until proteins reached the end of the stacking gel, and then at 100-140 V for 1-2 hours. Gels were transferred to PVDF membranes in transfer buffer (190 mM glycine 25 mM Tris, and 20% Methanol) for 2 hours at 400 mA. Membranes were stained with Ponceau in order to measure total protein loaded in each sample. After imaging, Ponceau was rinsed from the membranes and then membranes were blocked with 5% non-fat milk dissolved in TBST (25 mM Tris (pH 7.2), 150 mM NaCl, and 0.1% Tween 20) for 1 hour at room temperature. Membranes were incubated with primary antibody (VEGF and VEGFR2, Santa Cruz; AMPK, p-AMPK, and TSP-1 Cell Signaling Technology) in 5% non-fat milk dissolved in TBST at 4°C overnight. Membranes were washed with TBST for 10 minutes 3 times and then incubated in anti-rabbit or anti-mouse immunoglobulin G-horseradish peroxidase (Cell Signaling Technology) or fluorescence (Li-COR) conjugated secondary antibody for 1 hour. Membranes were again washed with TBST for 10 minutes 3 times and signals were visualized by fluorescence or chemiluminescence under FluorChem E system (protein simple).

Quantitative real-time PCR

RNA was isolated from muscle tissue samples using Trizol reagent (Invitrogen). First-strand cDNA was generated by random hexamer primers with MMLV Reverse Transcriptase (Invitrogen). Using a SYBR green PCR kit, the expression level of VEGF, VEGFR2, and TSP-1 was determined by real-time PCR reactions in the Roche LightCycler® 480 System (Roche). The internal control for gene expression was 18s expression. The primer sequences are listed below:

Table 2: Primer sequences for gene expression

Gene Name	Forward (5'-3')	Reverse (5'-3')
VEGF	AGGGCAGAATCATCACGAAGT	AGGGTCTCGATTGGATGGCA
VEGFR2	GTGATCGGAAATGACACTGGAG	CATGTTGGTCACTAACAGAAGCA
TSP-1	AGACTCCGCATCGCAAAGG	TCACCACGTTGTTGTCAAGGG
18s	GGCCCTGTAATTGGAATGAGTC	CCAAGATCCAACTACGAGCTT

2.3 Statistical Analysis

Data were analyzed by use of a two-way (2 groups × 3 time points) mixed-factorial analysis of variance (ANOVA) test. Following a significant *F* ratio, Fisher's LSD *post-hoc* analysis was performed. Significance was established at $\alpha = 0.05$ level and data reported as Mean ± SE.

CHAPTER 3. RESULTS

3.1 Subject Characteristics

Obese (OB) subjects had significantly greater body mass than lean (LN) subjects (OB: 36.6 ± 1.8 kg/m^2; LN: 21.7 ± 0.8 kg/m^2). LN subjects demonstrated a significantly higher VO_{2Max} than OB (LN: 37.3 ± 2.7 ml O_2/kg/min; OB: 26.1 ± 2.7 ml O_2/kg/min). OB demonstrated a higher 1 RM for single leg knee extension (LN: 27.8 ± 0.7 kg; OB: 40.3 ± 4.7 kg); however, when corrected for body mass, the difference was no longer statistically significant (P = 0.17). No differences were observed in fasting glucose between groups (LN: 4.8 ± 0.1 mM; OB: 4.9 ± 0.2 mM); however OB demonstrated a higher fasting insulin (LN: 11.8 ± 1.3 µU/ml; OB: 28.3 ± 4.8 µU/ml) and fasting HOMA-IR (LN: 2.46 ± 0.29; OB: 6.38 ± 1.23).

3.2 VEGF and VEGF receptor expression

The VEGF mRNA and protein responses to resistance exercise in LN and OB subjects are displayed in Figure 3. Exercise significantly increased VEGF mRNA similarly at 3 HR in both LN and OB subjects. There was no differences in VEGF protein between LN and OB PRE, 15 MIN or 3 HR post exercise.

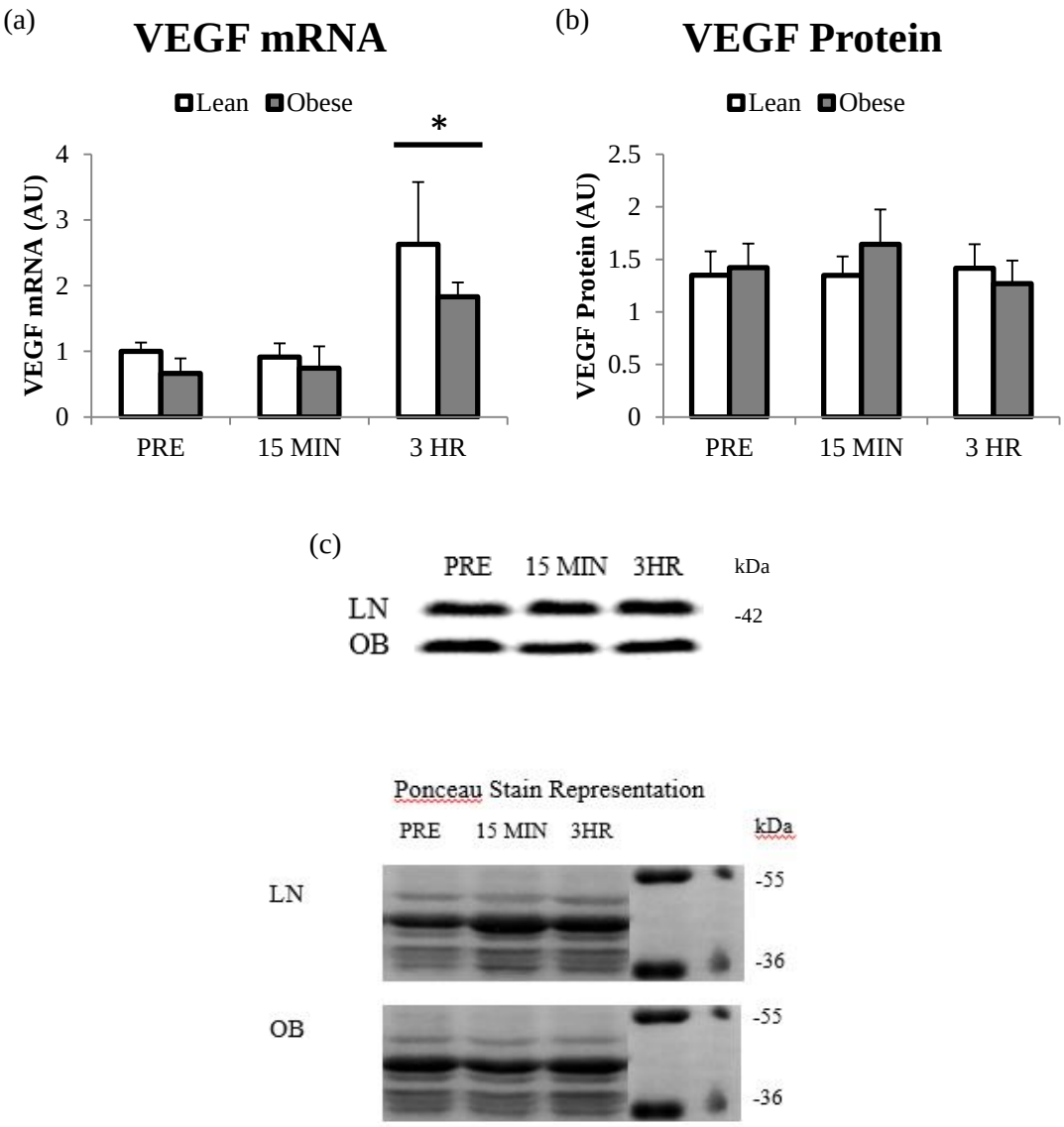

Figure 3. Skeletal muscle vascular endothelial growth factor (VEGF) mRNA (a) protein (b) and (c) western blot analysis in response to acute resistance exercise in LN and OB individuals. VEGF mRNA was significantly increased at 3 HR. *Significantly different from PRE ($P \leq 0.05$). Mean ± SE. n = 8 subjects/group.

Skeletal muscle VEGFR2 mRNA and protein responses to exercise in LN and OB individuals are displayed in Figure 4. No differences in the VEGFR2 mRNA or protein were found between LN and OB individuals PRE, 15 MIN or 3 HR post exercise. Exercise did not affect VEGFR2 mRNA or protein in either group.

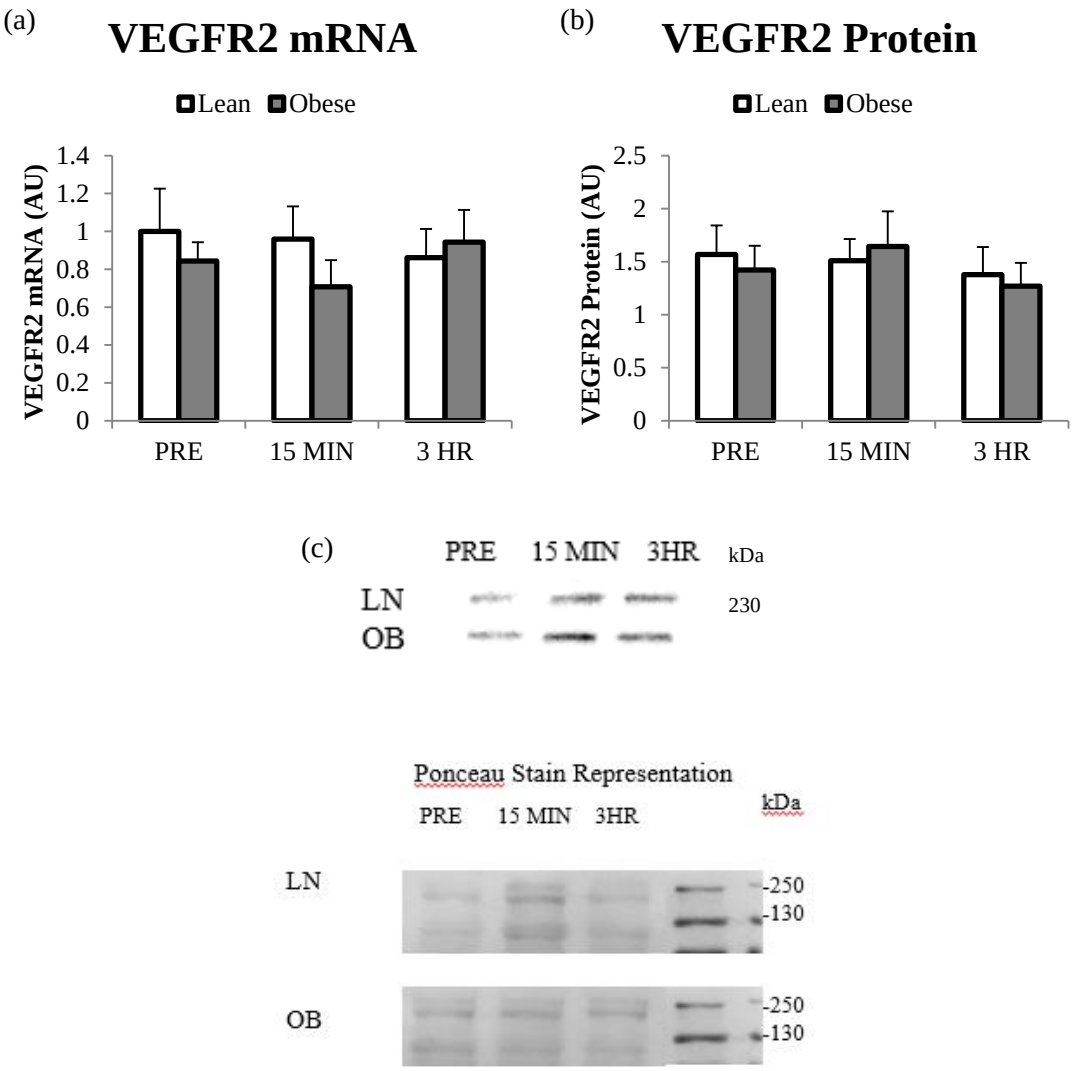

Figure 4. Skeletal muscle vascular endothelial growth factor receptor (VEGFR2) mRNA (a) protein (b) and western blot analysis (c) in response to acute resistance exercise in LN and OB individuals. Mean ± SE. *n* = 8 subjects/group.

3.3 TSP-1 expression

Skeletal muscle TSP-1 mRNA and protein responses to exercise in LN and OB individuals are displayed in Figure 5. There were no differences in TSP-1 mRNA between LN and OB at any time point and no effect of exercise. Similarly, there was no effect of obesity or exercise on TSP-1 protein expression.

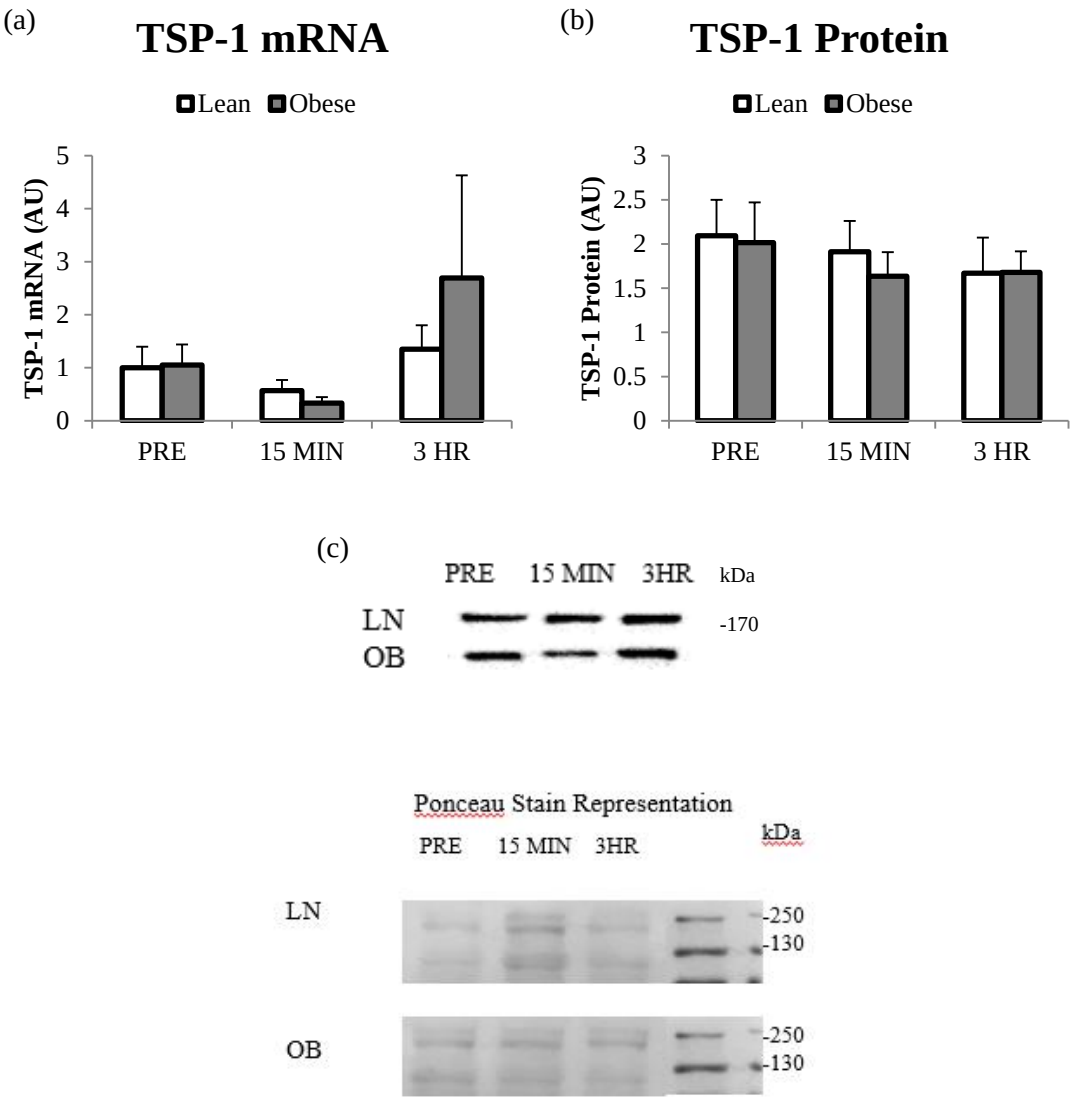

Figure 5. Skeletal muscle thrombospondin-1 (TSP-1) mRNA (a) protein (b) and western blot analysis (c) in response to acute resistance exercise in LN and OB individuals.

3.4 AMPK and p-AMPK expression

Skeletal muscle AMPK and p-AMPK protein responses to exercise in LN and OB individuals are displayed in Figure 6. Exercise and obesity had no effect on p-AMPK, pan-AMPK, or the p-AMPK/pan-AMPK ratio PRE, 15 MIN or 3 HR post exercise.

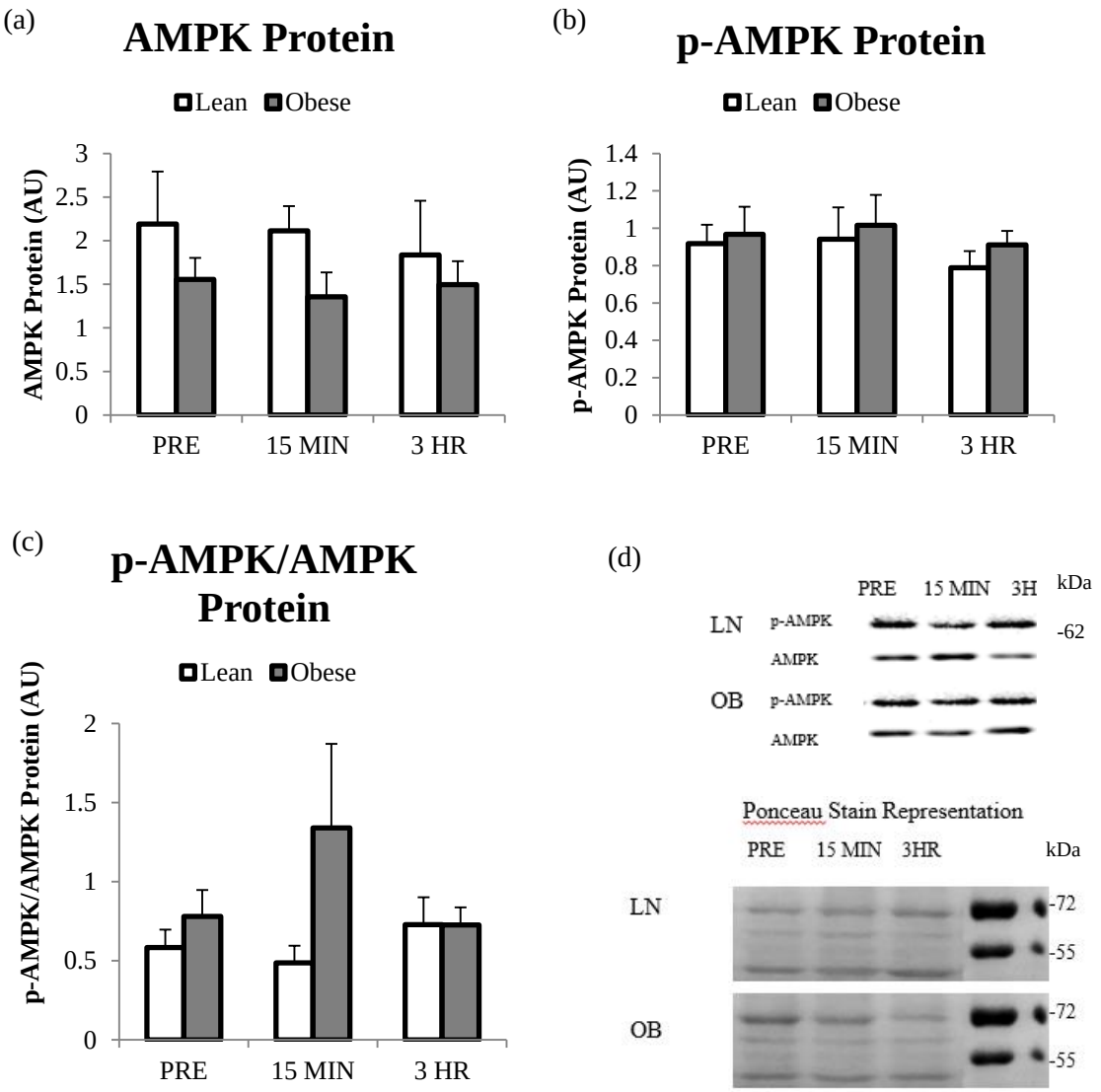

Figure 6. Skeletal muscle 5' AMP-activated protein kinase (AMPK) protein (a), phosphorylated AMPK (p-AMPK) protein (b) p-AMPK/AMPK protein (c) and western blot analysis (d) in response to acute resistance exercise in LN and OB individuals. Mean ± SE. *n* = 8 subjects/group.

CHAPTER 4. DISCUSSION

The principal findings of the current study are there were no differences in the expression of VEGF, VEGF receptor and TSP-1 at rest and following acute resistance exercise between lean and obese groups. Consistent with previous work, resistance exercise did increase VEGF mRNA in both lean and obese individuals. In addition, there was no difference at rest or in response to exercise in AMPK between lean and obese groups and acute resistance exercise did not increase the phosphorylation of AMPK in either group 15 MIN or 3 HR post exercise.

4.1 Obesity and basal VEGF and VEGF receptor expression

Similar basal levels of VEGF and VEGF receptor mRNA between the skeletal muscle of lean and obese individuals have been previously reported (Gavin et al, 2005). No there was no difference in the VEGF and VEGF receptor protein content of the skeletal muscle of lean and obese individuals (Gavin et al, 2005). Our data further support the notion that VEGF and VEGF receptor mRNA and protein expression are similar between lean and obese individuals at rest.

4.2 Obesity, exercise and VEGF and VEGF receptor expression

The current report utilizes a single bout of resistance exercise, a stimulus of muscle hypertrophy when repeated, in order to investigate the reduced capillary density observed with basal hypertrophy seen in obese individuals. Investigations using animal models suggest the skeletal muscle of obese have the ability to hypertrophy but have a decreased response to functional overload, a model for resistance exercise, compared to lean (Katta et al, 1985; Paturi et al, 2010). Similarly, Donnelly and colleagues showed an increased hypertrophy with resistance exercise in obese subjects but did not compare these results to lean counterparts (Donnelly et al, 1993). Therefore, this could result in lesser angiogenesis in obese individuals compared to lean due to less activation of hypertrophic pathways.

Very little research regarding the angiogenic response to resistance exercise in lean individuals has been conducted. Previous investigations show elevations in VEGF mRNA as early as 1 h following the completion of acute resistance exercise in lean individuals (Gavin et al 2007; Silvennoinen et al, 2015; Trenerry et al, 2007). Increases in VEGF mRNA >2.5 fold have been reported in human subjects (Gavin et al, 2007). This is relatively similar to what is shown in the present study, demonstrating an increase of ~2.2 fold 3 h post exercise. VEGF protein expression did not change following exercise, and no differences were found between lean and obese subjects. The present findings are in contrast to a previous report of increased VEGF protein expression by ~15% 2 h post resistance in lean individuals (Gavin et al, 2007). However, differences in analysis techniques could account for the variations, as Gavin and colleagues used ELISA for analysis which is thought to be more sensitive than Western Blot techniques (Kuijf et al,

2009). Further investigations are needed in order to develop an accurate time course of VEGF protein expression in the skeletal muscle following resistance exercise.

Increases in VEGF receptor mRNA have been observed at 4 h but not 2 h post resistance exercise in lean individuals (Gavin et al, 2007). Considering other factors involved in the present study, 3 h was chosen for the final biopsy with the hopes this would be sufficient time for changes in VEGF receptor mRNA to occur. The present study shows no increase in VEGF receptor mRNA in lean or obese individuals up to 3 h following resistance exercise. Similarly, exercise did not increase skeletal muscle VEGF receptor protein expression in lean or obese subjects. Gavin and colleagues also showed no changes in VEGF receptor protein expression 4 h post resistance exercise in lean individuals, as expected since protein changes occur after mRNA changes (Gavin et al, 2004). However, VEGF receptor protein expression increases have been observed 6 hours post aerobic exercise (Gustafsson et al, 2005). Since little is known about other factors involved in this study, a 6 h instead of 3 h post biopsy could have resulted in potentially missing important changes immediately following the acute resistance exercise bout. The last skeletal muscle biopsy was obtained 3 h post exercise in the present study. Therefore, 3 h may be insufficient to observe changes in VEGF receptor mRNA or protein expression following acute resistance exercise.

4.3 Obesity and basal TSP-1 expression

TSP-1 is an angiostatic factor that is present in several tissues and is involved in cell adhesion, migration, and growth factor regulation (Lawler, 2002; Roberts, 1996; Sage & Bornstein, 1991). Comparisons of TSP-1 levels between lean and obese have

previously been investigated in the adipose tissue, as dynamic alterations in the extracellular matrix are required during the expansion of adipose tissue (Kong, Cavalera & Frangogiannis, 2014; Sun, Kusminski & Scherer, 2011). Vascularization and adipose tissue development are coupled from early development throughout adult life (Cranell, Hausman & Kral, 1997) Adipocytes play an important role in guiding and maintaining microvessel growth (Lau, 1990; Lau et al, 1990) and are a major site of TSP-1 secretion (Varma et al, 2008). Animal models demonstrate the lack of TSP-1 or its receptors as a protective mechanism against the development of obesity (Frazier et al, 2011; Kong et al, 2013; Maimaitiyiming et al, 2015). However, it has also been proposed that TSP-1 deficiency only improves glucose tolerance and increases insulin sensitivity with no effect on the development of obesity (Li et al, 2011). TSP-1 mRNA expression in the adipose tissue of human subjects suggests a positive correlation between TSP-1 expressed in the visceral adipose tissue and BMI (Matsuo et al, 2015; Varma et al, 2008). Evidence of increased TSP-1 expression in obese is also observed in other organs such as the heart (Gonzalez-Quesada et al, 2013) and blood vessels (Stenina et al, 2003).

To our knowledge, no previous studies have reported basal levels of TSP-1 expressed in the skeletal muscle of lean compared to obese individuals. Chronic delivery of TSP-1 to the skeletal muscle reduces capillarity, as shown in mouse models (Audet, Fulks, Stricker & Olfert, 2013). However, results of the present study show no differences in basal skeletal muscle TSP-1 mRNA despite significant differences in BMI. Therefore, another angiostatic factor may be responsible for the reduced capillary density present in the skeletal muscle of obese individuals.

4.4 Obesity, exercise and TSP-1 expression

Investigations of TSP-1 mRNA expression following exercise have been reported with lean subjects. In Wistar rats, TSP-1 mRNA expression is increased 3.5 fold immediately following aerobic exercise, with further elevations 1 h post exercise before returning to baseline (Olfert, Breen, Gavin & Wagner, 2009). Three days of aerobic training attenuates this increase while the acute response is restored following 8 wks of training (Olfert, Breen, Gavin & Wagner, 2009). TSP-1 mRNA demonstrates a similar response to aerobic exercise in lean human subjects (Hoier et al, 2012). The current study is the first to investigate the TSP-1 mRNA and protein expression following acute resistance exercise in lean and obese subjects.

Aerobic exercise increases AMPK activity and TSP-1 expression at similar time points, within 2 h post exercise (Hoier et al, 2012; Olfert, Breen, Gavin & Wagner, 2009; Olfert et al, 2006; Olfert, Wagner, & Power, 2000; Sriwijitkamol et al, 2007; Stephens et al, 2002); however the present study shows no increase in AMPK phosphorylation or TSP-1 expression following resistance exercise. Though a negative finding, this is consistent with TSP-1 expression regulation by AMPK. Further investigation into skeletal muscle TSP-1 expression following exercise may be beneficial in order to investigate potential regulators of TSP-1 and establish an accurate time course of response.

4.5 Obesity and basal p-AMPK and AMPK expression

The phosphorylation of AMPK regulates cellular energy usage by increasing the activity of energy conserving pathways while concurrently reducing the activity of

energy consuming pathways (Hawley et al, 1995). In obese Zucker rats, basal levels of skeletal muscle AMPK phosphorylation and the subsequent p-AMPK:AMPK ratio are significantly higher than their lean counterparts (Chen, Lee, Kuo & Ho, 2011). Other investigations, including the present study, have found no effect of obesity on basal AMPK activity (Lee-Young et al, 2011; Steinberg et al, 2004). Differences between human and animal models could in part account for the conflicting results. More investigation into the basal activity of AMPK between lean and obese, especially in human subjects, is needed.

4.6 Obesity, exercise and p-AMPK and AMPK expression

AMPK is an important regulator of the body's response to stressors. One important stressor known to increase AMPK activity is exercise (Winder & Hardie, 1996). Mouse models have demonstrated that diet-induced obesity has a negative impact on AMPK activity in response to acute aerobic exercise (Lee-Young et al, 2011). Similar results have been shown in human studies involving moderate intensity aerobic exercise in obese subjects (Sriwijitkamol et al, 2007). In contrast, lean and obese Zucker rats show no difference in the activation of AMPK following 6 wks of aerobic exercise training (Chen, Lee, Kuo & Ho, 2011). However, obese Zucker rats have significantly lower AMPK phosphorylation following exercise training, while lean Zucker rats show no change (Chen, Lee, Kuo & Ho, 2011). This suggests aerobic exercise training could restore the AMPK response of obese subjects.

Investigations into AMPK activity in response to resistance exercise in lean individuals demonstrate conflicting results (Ahtiainen et al, 2015; Apro et al, 2015;

Dreyer et al, 2006; Silvennoinen et al, 2015). However, the phosphorylation of AMPK appears to be resistance exercise volume-dependent. Ahtiainen and colleagues observed an increased p-AMPK protein expression 30 min following 10 but not 5 sets of 10 RM using a leg press device (Ahtiainen et al, 2015). Previous reports suggest AMPK activity is increased within the first 30 min of exercise (Dreyer et al, 2006; Silvennoinen et al, 2015). The present study observed no changes in p-AMPK following 3 sets of knee extension. Therefore, the volume of the current exercise bout may have been insufficient to elicit a response since samples were obtained within the suggested time window.

4.7 Limitations

As with all studies, limitations to the experimental design are present in the current report. Participants were both male and female and the menstrual cycle of female subjects were not controlled for. Hormonal differences could impact the skeletal muscle and therefore could result in increases variability within the results. Participants documented, but did not alter, their diet in this experiment. Subjects were asked to eat normally and record all food and beverage intake for three days preceding Day 2. Hours of sleep prior to Day 2 procedure were not controlled. At least two weeks separated Days 1 and 2; however the number of days between varied between subjects due to scheduling availability. Baseline biopsies were obtained from the non-working leg to avoid three biopsies on one single leg. Time of biopsies attempted to capture changes in muscle mRNA and proteins with limited information on their time courses following resistance exercise. Therefore, potential changes could have been missed. Due to limited information on the angiogenic response following resistance exercise, duration and intensity of the exercise bout may have been insufficient to produce changes.

4.8 Future Directions

The increased VEGF expression reported in the current study further supports findings that resistance exercise training can elicit angiogenesis, though there is no difference between lean and obese individuals. However, the skeletal muscle of obese individuals is unable to maintain capillary density with muscle hypertrophy. It would be beneficial to study the long term angiogenic response to high volume resistance training in obese individuals, as there appears to be a disruption between the acute response and the development of capillaries. Other possible angiostatic factors that could be hindering capillary formation should be investigated.

CHAPTER 5. CONCLUSION

In summary, the present study demonstrates that lean and obese individuals have a similar angiogenic response to acute resistance exercise. Future studies are needed in order to observe the effect of repeated bouts of resistance exercise on skeletal muscle angiogenesis between lean and obese individuals. Considerations into possible angiostatic factors other than TSP-1 contributing to reduced angiogenesis should be investigated.

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